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The Relationship Between Subunit Composition and O_2 Binding of Blue Crab Hemocyanin

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Abstract. Hemocyanin-O_2 affinity differs in estuarine and seaside populations of the blue crab *Callinectes sapidus.* The difference, which is adaptive, is accompanied by different proportions of the six subunits that make up the native hemocyanin polymers. When the hemocyanins are dissociated and separated by alkaline electrophoresis, six subunits can be resolved in most estuarine individuals but two of the six are either present in low concentrations in or absent from most seaside individuals. A third subunit is also variable but the variation is not clearly correlated with locality. O_2 binding measurements of Hcs with the two major wild phenotypes but collected at the same locality reproduce the difference between the natural populations. Measurements on several intermediate phenotypes suggest that the variation of one of the subunits is more important physiologically than variation of the other two.

Introduction

Mason *et al.* (1983) found that estuarine and seaside populations of the blue crab *Callinectes sapidus* Rathbun, which are separated by about 200 km, have different intrinsic O_2 affinities of their hemocyanins (Hcs). The differences ensure adequate O_2 transport in each locality despite large changes in blood levels of several allosteric effectors (Mangum, 1986). After eight days estuarine animals transferred to high salinity had a HcO_2 affinity indistinguishable from the seaside phenotype, and seaside animals transferred to low salinity exhibited a shift in HcO_2 affinity towards the estuarine phenotype. Cooperativity did not change and the response of the Bohr shift was not examined.

Although Mason *et al.* (1983) did not reach a firm conclusion concerning the factor responsible for the acclimation, they did report a difference in the Hc subunit composition of pooled samples taken from six to seven experimental animals representing each locality. While six subunits were clearly present in the estuarine animals immediately after collection and still acclimated to estuarine conditions, only four were detected in seaside animals immediately after collection and still acclimated to seaside conditions. Perhaps most important, a shift in Hc subunit composition towards the seaside phenotype accompanied the shift in O_2 affinity following transfer of estuarine animals to high salinity. (The sample from the seaside group transferred to low salinity was unavailable for analysis.) Hc concentration rose in animals transferred from high to low salinity and fell in animals transferred from low to high salinity. This is consistent with a hypothesis of net synthesis of selected subunits at low salinity and net degradation at high salinity. Mason *et al.* (1983) tentatively advanced the hypothesis that the acclimation might result from rearrangement of the proportions of functionally distinct subunits, a mechanism long the subject of conjecture (*e.g.*, Bonaventura and Wood, 1980).

A preliminary examination of the phenotypes of 51 individuals from each of the two localities revealed variability of a third subunit which is not clearly correlated with salinity. A detailed examination of the distribution of the various phenotypes is now under way ([illegible] *al.*, 1985).

To determine the subunit composition [illegible] individual crabs as well as O_2 affinity, we attemp[illegible] collect larger volumes of blood while repeating the transfer experiment. Our attempts met with limited success due to the greater mortality that accompanied heavier bleeding. Therefore, we performed somewhat less direct but also less invasive experiments designed to test the hypothesis that the Hc phenotypes observed in estuarine

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and seaside crabs are responsible for the differences in HcO_2 affinity. Because HcO_2 affinity also changes with season (Mauro and Mangum, 1982), we performed our experiments during July—the same month in which previous experiments were performed (Mason *et al.*, 1983).

Materials and Methods

We either captured or purchased crabs from local watermen whose pots had been placed at known locations. Water salinity was determined, by conductivity (Yellow Springs Instrument Co. Model 33 salinometer), in both cases.

As in the previous investigation (Mason *et al.*, 1983), a transfer experiment was designed as paired observations on the same individuals before and after a salinity change. Blood was taken from each individual before and after the transfer. We intended to save one aliquot for electrophoresis and use the other for O_2 binding. Poor survival dictated otherwise and we used all of the material available to ascertain our earlier finding of a shift in HcO_2 affinity.

As an alternative to holding previously bled animals in the laboratory at the time of year when mortality is already great, we observed different phenotypes at the estuarine (York River) and seaside (Wachapreague) habitats. We first sampled large numbers of animals, then identified the phenotypes electrophoretically, and finally performed O_2 binding measurements on them.

HcO_2 equilibrium measurements were made using the cell respiration method (Mangum and Lykkeboe, 1979). After clotting the samples were homogenized in a tissue grinder, centrifuged, and dialyzed overnight against a saline.

Regression lines were fit to the data describing log P_{50} as a function of pH and the 95% confidence intervals around the lines and their slopes were compared for overlap. Values for P_{50} at a single pH and those for cooperativity were analyzed according to Student's *t*-test.

Hcs were dissociated by dialysis against 0.05 *M* Tris HCl (pH 8.9) for 12–16 h. In the early experiments polyacrylamide gel electrophoresis was performed as indicated by Mason *et al.* (1983). Later the gels were prepared according to the method of Hames and Rickwood (1985), which gave better separations. The two protocols differ only in the concentrations of buffer, crosslinker, and TMED (N,N,N′,N′-tetramethylethylenediamine). All of our gels (0.75 mm × 16 cm) consisted of a 3% stacking gel over a 12.5% resolving gel. A discontinuous buffer system was used, with Tris-glycine buffer (pH 8.1) in the upper chamber and Tris-HCl buffer (pH 8.9) in the lower chamber. Best separation was obtained when the electrophoresis proceeded for 8–12 h at constant current (15–18 mA). The gels were stained for 40 min with 0.1% Coomassie blue (G-250) in 25% trichloroacetic acid and destained with a mixture of acetic acid and methyl alcohol.

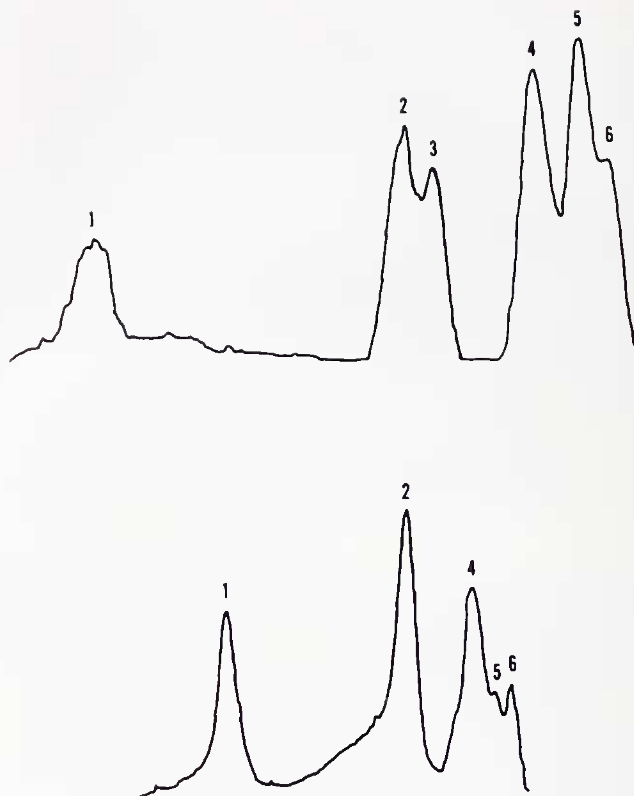

Figure 1. Densitometer scans of Hc wild phenotypes. Top: estuarine population (10 cm/min scan); bottom: seaside population (2 cm/min scan).

Results

Here and in the Discussion below we designate the electrophoretic phenotypes as follows. Maximal concentrations of all six subunits was the predominant phenotype in our preliminary sample from the upper York River estuary of Virginia, where the typical salinity range is 3–16‰. This "estuarine wild phenotype" is symbolized as HHHHHH (Fig. 1). Minimal concentrations or absence of subunits 3 and 5, was the predominant pattern at Wachapreague, Virginia, where the salinity normally ranges from 29 to 33‰. This "seaside wild phenotype" is symbolized as HHLHLH (Fig. 1). Additional phenotypes examined here include minimal concentrations of subunits 3 and 6 together (HHLHHL), subunit 3 alone (HHLHHH), and subunit 6 alone (HHHHHL). Still another phenotype (HHLHLL) has been recovered on occasion, but not in the samples examined here.

Each of three attempts to collect large volumes of blood while repeating the transfer experiment met with

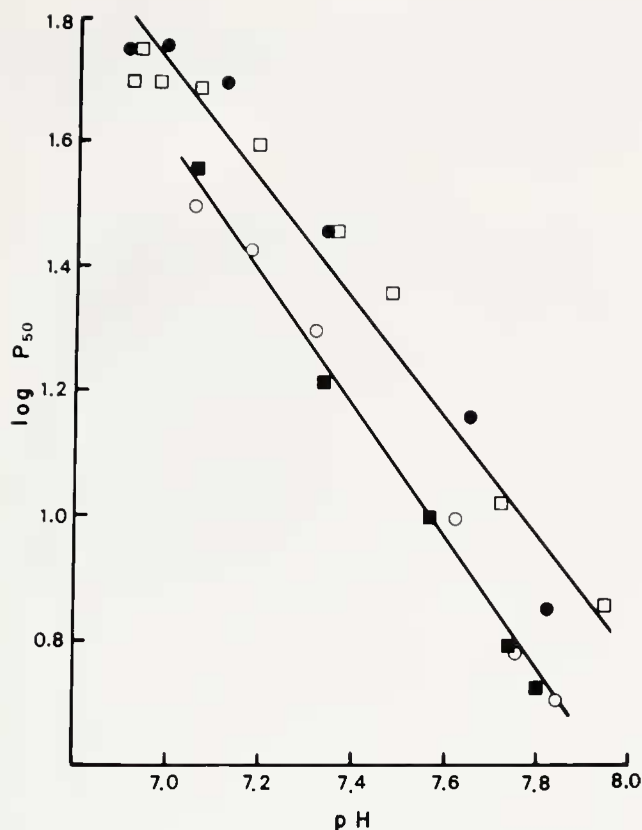

Figure 2. Relationship between pH and P_{50} of Hcs dialyzed against 0.05 *M* Tris maleate buffered seawater (32‰), 25°C. (●) Blood from estuarine crab taken immediately after collection. (○) Blood from same crab held 10 days at 32‰. (■) Blood from seaside crab immediately after collection and (□) blood from same crab after 11 days at 5‰.

little success due to mortality, a common problem at summer temperatures. In the first two attempts all 14 animals in each group were dead by the end of 5 days. The O_2 binding properties of the bloods of several of these animals examined had not changed significantly during the acclimation period.

In the third attempt a single individual (of 21) in each group survived. Of those animals transferred from 32 to 5‰, one individual survived for 11 days, and of those transferred from 8 to 32‰, one individual survived for 10 days. Daily feeding (shrimp) and/or a two-step transfer did not enhance survival. The O_2 binding measurements made at physiological pH on samples from the two populations (Fig. 2) agree with the trends reported earlier, as do those made 10–11 days following transfer to the alternative salinity. We should note that the absolute values in these data are slightly different, most likely because the unavailability of chemicals at our seaside laboratory made it necessary to dialyze the samples against seawater rather than a strictly physiological saline. The difference between the two populations is also slightly greater than observed before or since, possibly due to different inorganic ion sensitivities of the two phenotypes. Regardless, HcO_2 affinity in the sole survivor of the transfer in either direction is indistinguishable from that of the alternative population. The present results show no difference in the Bohr shift.

We did not regard results from a single animal as an adequate test, so we adopted a new approach. Forty-five animals were caught at various locations in the York River estuary and its tributaries; animals with the two wild phenotypes were identified. Large blood samples were taken without regard for survival, and divided. One aliquot was electrophoresed and, simultaneously, another aliquot was used for O_2 binding. Due to the large number of samples analyzed and the small number with the seaside phenotype recovered, the observations were confined to physiological pH. The results are shown in Table I. There is clearly a difference ($P < .001$) between the estuarine (collected at 5‰) wild phenotype and the seaside wild phenotype, which was collected on this occasion at low salinity (2‰).

In our final experiment we sampled the seaside population (n = 55) which, at least occasionally, has proven to be more diverse than estuarine populations and, since each chain appears to vary independently of the others, presented the possibility of yielding intermediate as well as both wild phenotypes. On this occasion we identified the phenotypes prior to the O_2 binding measurements so that we could discard unneeded duplicates and make more extensive observations on any one. The measurements, which were completed within 5 days of sample collection, indicate that at physiological pH the difference in the P_{50} values (antilog) for the two wild phenotypes, both collected at the seaside locality (32‰), is 30–35% (Fig. 3). This is the same as the difference between the two wild phenotypes collected at their respective natural localities (Mason *et al.*, 1983).

This set of observations appears to show that the absolute difference between the two wild type phenotypes diminishes when P_{50} becomes very small above pH 7.8, a range not examined earlier. However, the data for intermediate phenotypes (Fig. 4) suggest that this tendency may not be real. More importantly, in the 7.0–7.8 pH range data for the intermediate phenotypes lie between

Table I

O_2 binding of Hc phenotypes found in sample of estuarine blue crabs[1]

Phenotype	pH	P_{50}	n_{50}
HHHHHH	7.50	21.8 ± 0.7 (6)	3.79 ± 0.34 (6)
HHLHLH	7.53	15.2 ± 0.3 (4)	3.16 ± 0.25 (4)

[1] Dialyzed against buffered saline described in legend of Figure 2. 25°C. Mean ± S.E. (N).

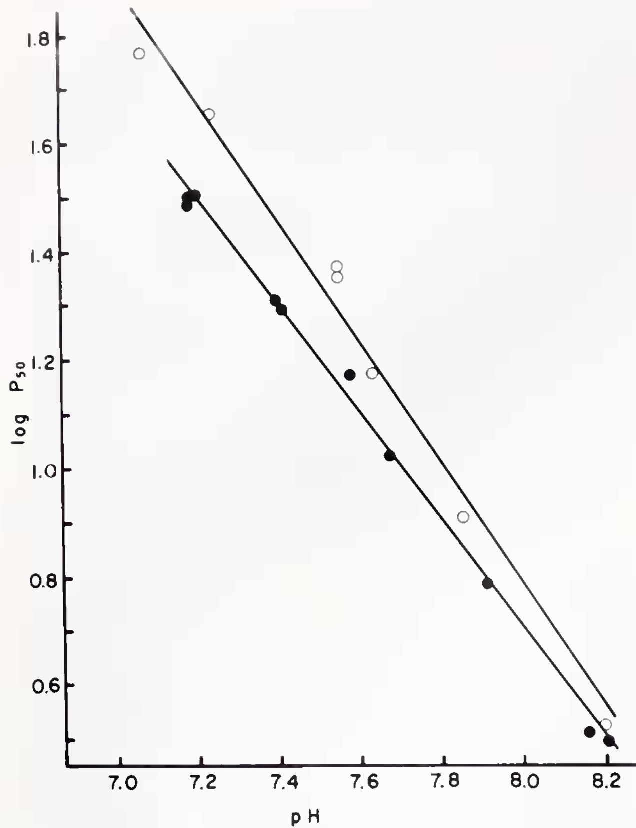

Figure 3. Relationship between pH and P_{50} of samples taken from one seaside crab with HHHHHH phenotype (○) and three seaside crabs with HHLHLH phenotype (●). Dialyzed against 0.05 *M* Tris maleate buffered saline containing 368 m*M* NaCl, 12 m*M* KCl, 18 m*M* $CaCl_2$, 21 m*M* Na_2SO_4, 17 m*M* $MgCl_2$, and 3 m*M* $NaHCO_3$ (Mason *et al.*, 1983). 25°C. The lines fitted by regression analysis differ at $P < .001$.

those for the two wild phenotypes (Fig. 4). When subunit 6 alone drops from high to low concentrations (HHHHHH to HHHHHL), HcO_2 affinity also decreases; the regression line describing the data differs from that for the estuarine wild type throughout the pH range examined. It also differs from the seaside wild phenotype (HHLHLH) throughout the entire pH range. However, when band 3 alone is low (HHLHHH), HcO_2 affinity differs very little from that of the seaside wild phenotype (HHLHLH). The differences are significant only at the pH extremes (7.0–7.2 and 8.2). Moreover, the HHLHHH phenotype differs from HHHHHH (estuarine wild phenotype) throughout the entire pH range. A decrease in concentration of subunit 6 as well (HHLHHL) has little or no effect: the data for this phenotype differ from those for HHLHHH only slightly and only at pH 7.0–7.4. They differ from HHHHHH throughout the pH range. The differences are summarized in Table II and the O_2 affinities at physiological pH are specified in Table III. Like Mason *et al.* (1983), we found no differences in cooperativity between any of the phenotypes (P = .15–.82); the magnitude of the Bohr shift (described by slopes of the regression lines) also failed to differ significantly (P > .05).

Discussion

The role of subunit heterogeneity in the assembly of native Hc polymers is rapidly being elucidated (Markl, 1986; Markl *et al.*, 1986; Stöcker *et al.*, 1986). Using mixtures of purified subunits Stöcker *et al.* (1986) found that the maximum amount of assembly to the native dodecamers of *C. sapidus* Hc is achieved only when all six subunits are present in approximately physiological ratios. To clarify this comparison we should note that Stöcker *et al.*'s (1986) order of numbering the bands is the opposite of ours, viz. it progresses from anodic to cathodic rather than the other way around as practiced here and also by Mason *et al.* (1983) and Johnson *et al.*

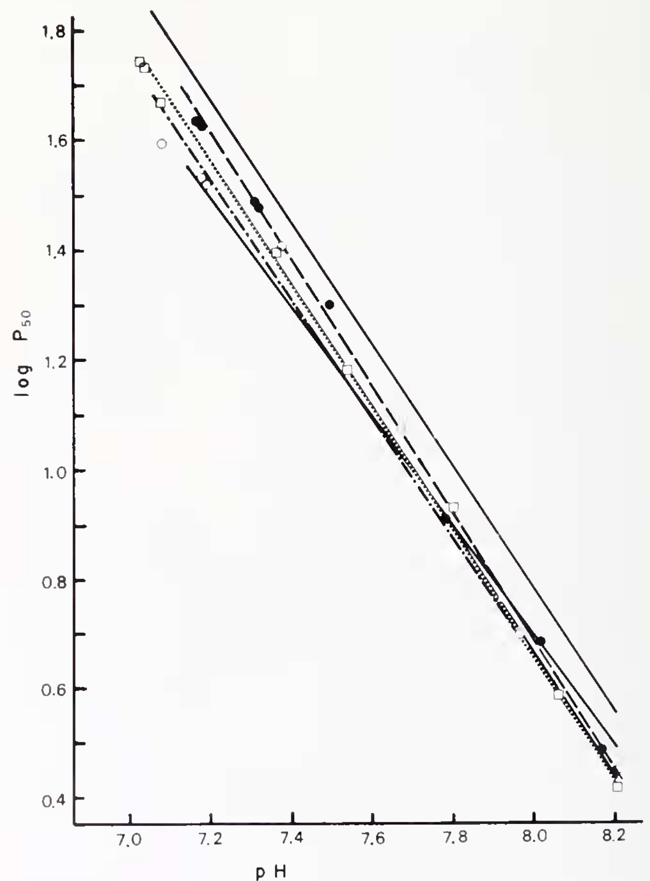

Figure 4. Relationship between pH and P_{50} of samples from two seaside crabs with HHHHHL phenotype (●, dashed regression line), three with HHLHHH phenotype (○, dashes and dots), and two with HHLHHL phenotype (□, dotted line). Dialyzed against buffered saline described in legend of Figure 2. 25°C. The solid lines reproduced from Figure 2 describe the two wild type phenotypes.

Table II

Differences in HcO_2 affinities of the five phenotypes recovered in Wachapreague in 1986

	HHLHLH	HHLHHL	HHLHHH	HHHHHL	HHHHHH
HHLHLH	—	*	*	**	**
HHLHHL	*	—	*	**	**
HHLHHH	*	*	—	*	**
HHHHHL	*	**	*	—	**
HHHHHH	**	**	**	**	—

** Significant (no overlap of 95% confidence intervals around regression lines in Figs. 2 and 3) throughout pH range examined.
* Significant only in limited pH range. See text for details.

(1984). We should also note that the gel scans shown by Stöcker *et al.* (1986) appear to us to represent the estuarine wild phenotype in that all six bands are easily detectable, although comparison is somewhat difficult since they used the crossed immunoelectrophoresis technique.

Based on a combination of characteristics derived from electrophoretic mobility, the immunological response, antigenic sufficiency, and the role in protein assembly, Markl (1986) and Markl *et al.* (1986) devised a scheme of classifying the arthropod Hc subunits. In their terminology the three variable subunits in *C. sapidus* are one alpha prime (our subunit 3), one alpha (subunit 5), and one gamma (subunit 6) chain and the invariant (at least thus far) subunits are two betas (1 and 4) and one alpha (2).

Native blood in this species contains a mixture of hexamers and dodecamers in a ratio reported to be anywhere from 1:4 (Hamlin and Fish, 1977; Johnson *et al.*, 1984) to as much as 1:1, possibly due to genuine physiological variation (Herskovits *et al.*, 1981). Any one of the three kinds of chains is competent to form hexamers but the maximum yield is obtained when small amounts of gamma chains are added to purified preparations of alpha chains (Stöcker *et al.*, 1986). The second step in assembly of dodecamers, a physiologically important process (Snyder and Mangum, 1982; Mangum, 1986), requires the alpha prime chains, which are believed to serve as the interhexamer bridgers. Although one of the two alpha chains (subunit 5) in *C. sapidus* is frequently low or absent in seaside crabs, the apparent invariance of a second alpha chain (subunit 2) may ensure the formation of at least hexamers. The variability of alpha prime chains, believed to play an integral role in further assembly, however, raises the question of whether a changing ratio of hexamers to dodecamers is responsible for the differences in HcO_2 affinity observed here.

Table III

O_2 affinities at pH 7.6 and 25°C of Hc phenotypes collected from seaside population

Phenotype	P_{50}	95% confidence interval
HHHHHH	16.7	16.4–17.1
HHHHHL	14.3	14.2–14.3
HHLHHL	12.9	12.8–12.9
HHLHHH	12.5	12.4–12.5
HHLHLH	12.6	12.5–12.6

Data from Figures 3 and 4.

Johnson *et al.* (1984) reported that the hexameric fraction found in the blood of *C. sapidus* lacks subunit 5, one of the alpha variables, and subunit 6, the gamma variable. Since subunits 2 and 3 were not separated it is not clear whether Johnson *et al.*'s (1984) scans of dodecamers resemble those of our HHLHHH, a not especially common phenotype, or HHHHHH, the estuarine wild phenotype. Johnson *et al.* (1984) mentioned that the O_2 affinities of hexamers and dodecamers are similar, although no data are shown. According to their scans hexamers should have either a HHHHLL phenotype, which we have never recovered, or a HHLHLL phenotype, which is very rare and for which we have no O_2 binding data. However, if, as suggested above, the variability of subunit 5 is of little or no physiological importance, then the O_2 binding of HHHHLL should resemble that of HHHHHL, which clearly differs from the two wild phenotypes although its relationship to the rare HHLHLL remains unknown.

Herskovits *et al.* (1981) implied that the observed range in the ratio of hexamers to dodecamers was correlated with season. Due to the potential importance in relation to the present findings and also to the seasonal difference in O_2 binding (Mauro and Mangum, 1982), a thorough investigation of the O_2 binding behavior of hexameric and dodecameric fractions would seem to be in order.

In any event, the present results confirm the earlier finding of differences in the respiratory properties of the HcO_2 transport system within the species *Callinectes sapidus*. Due to the paucity of the data, they less strongly

support the earlier inference that the adaptation is acclimatory and therefore non-genetic in origin. Indeed, none of our work, past or present, even pertains to the genetic status of the variability of subunit 6. But our present findings convincingly argue for a functional as well as a structural role of the various subunits, and a functional importance of phenotypic variability.

Acknowledgment

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